OBSERVATIONS ON SEROLOGY OF TUNA

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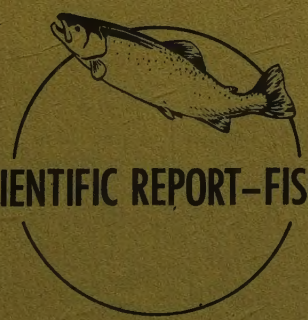

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EXPLANATORY NOTE

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United States Department of the Interior, Fred A. Seaton, Secretary
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OBSERVATIONS ON SEROLOGY OF TUNA

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ABSTRACT

The present paper presents observations showing that individual variations exist among the erythrocyte antigens of oceanic skipjack, and that species variations exist among other tunas. Further, data are presented concerned with techniques and antigenic specificities that might be profitably applied to racial studies in tunas and other fish.

OBSERVATIONS ON SEROLOGY OF TUNA

This paper describes observations and experiments concerned with the serology of tuna. The techniques and concepts used are similar to those employed in blood type studies (Cf. Mourant, A.E., 1954, Race, R.R., and R. Sanger, 1954; and Owen, R.D., C. Stormont, and M.R. Irwin, 1947). The antigens used in blood-typing have certain properties that make them peculiarly suited to comparative studies of subpopulations (also often termed races or breeding stocks) within single species.

These properties may be briefly outlined as follows. First, the presence or absence of a particular antigen on the red blood cells of an individual is genetically determined in a direct manner, uncomplicated by dominance or gene interaction (excepting only very rare instances). This means that the presence of an antigen on a red blood cell is direct evidence that the gene determining this antigen is present in the individual involved, while the absence of this antigen on the cells of another individual is direct evidence that the gene determining the antigen is also absent. In addition, the direct relation between gene and antigen is not influenced by variations in the environment that the individual may be subject to during its lifetime.

These properties, together with the highly sensitive yet relatively simple techniques employed in blood-typing, make the red-cell antigens particularly favorable indicators of genetic variation within populations. Statistical comparisons can therefore be made of the frequency of occurrence of selected antigens occurring among subpopulations. Such comparisons can reveal whether the subpopulations are alike or different with respect to the frequency of the antigens concerned, and therefore whether they are freely interbreeding and exchanging genes. In this way separate breeding stocks (races) may be detected through a study of red-cell antigens when other meristic characters are not available.

The success of blood-type studies that have been made on populations of humans, cattle, doves, chickens, and other warm-blooded animals led to the initiation of similar studies on fishes (Cf. Cushing, 1952a, b; Cushing and Sprague, 1952, 1953) with the aim of extending this work to where it might be employed in the study of problems of interest to fisheries. Of great interest in this connection are studies by Japanese biologists (Yamaguchi and Fujino, 1953; Fujino, 1953) who have been able to discover variations in the blood types of individuals within several species of whales, and who have made a start in the antigenic analysis of whale populations.

The major objectives of the work reported in the present paper have been to discover intraspecific individual variations in the red-cell antigens of tuna, to investigate the practicability of using samples of frozen whole bloods, and to develop serological techniques simple enough to be adapted to large-scale field investigations of interest to fisheries research and students of migration and evolution.

Research was conducted at Santa Barbara College with the assistance of grants from the Scripps Institution of Oceanography, and for three weeks (in July 1955) at the Hawaii Marine Laboratory, University of Hawaii, Honolulu. The visit to Hawaii was made possible by the Office of Naval Research, currently supporting in large part the author's research through a contract for the investigation of the serology of marine animals. Work with fresh intact tuna erythrocytes in Hawaii led to the discovery of individual antigenic variations in the oceanic skipjack and also permitted a consolidation of the Santa Barbara researches. George Durall is assisting in this research. In addition, Mrs. Elyse Beaver and former students Barbara Drake, Lucian Sprague, and Donald Shawhave contributed to various phases of the work at Santa Barbara.

The author is also indebted to the following persons and organizations: O.E. Sette of the Pacific Oceanic Fishery Investigations, Dr. M.B. Schaefer, Inter-American Tropical Tuna Commission, and E.K. Holmberg, Fish Commission of Oregon, for directing the collection of many samples of the frozen whole tuna blood. Vernon E. Brock, Division of Fish and Game, Board of Agriculture and Forestry, Honolulu, Hawaii, Dr. Robert Hiatt, Department of Zoology, University of Hawaii, Dr. Howard Buroughs, and the staff of the Hawaii Marine Laboratory assisted the author in many ways. The author is also indebted to Dr. Lionel A. Walford, U.S. Fish and Wildlife Service, and Dr. Carl L. Hubbs, Scripps Institution of Oceanography for much advice and encouragement during these studies. The collection and identification of tuna during the author's stay at the Hawaii Marine Laboratory was made possible through the assistance of the following men: Georges Gilbert, skipper of the Makua, research vessel of the Hawaiian Division of Fish and Game, Lester Zukeran, skipper of the Salpa, research vessel of the Hawaii Marine Laboratory, and Harry Yagi, owner and skipper of the aku boat Venus, Honolulu. Dr. Leon E. Mirmose of the Blood Bank of Hawaii contributed samples of human blood. Hyland Laboratories, Los Angeles, have donated human blood-typing serums. Dr. George Ridgway, U.S. Fish and Wildlife Service, contributed suggestions on the manuscript.

MATERIALS AND METHODS

The tuna bloods used in these studies were obtained either as frozen whole bloods transported to Santa Barbara by air, or as fresh bloods collected in the vicinity of Oahu. Bloods were taken from living fish by various methods such as heart puncture, tail bleeding, or cutting the conus arteriosus at its narrowest constriction beneath the gills. The author's experience was that the most practical method was the latter, cutting the vessel with a knife and collecting the blood in a wide mouth screw-cap glass bottle (plastic is recommended for future work). Twenty five to 100 milliliters of blood could readily be obtained in this way from fish averaging two to three feet in length.

In addition to the frozen and fresh bloods studied, a collection of nine skipjack bloods was taken from fish being unloaded at the dock after a day's fishing. These fish had been dead and iced for approximately 6 to 10 hours. The bloods taken were kept at refrigeration temperatures for approximately 36 hours or longer while the author went collecting fresh bloods on the Venus. This series of bloods showed varying degrees of hemolysis, but eight of the nine samples upon washing yielded suspensions of erythrocytes that appeared in excellent condition.

For reason of time, further study of this material from dead fish was dropped in favor of working on fresh cells, but the observations just reported suggest that it may be feasible to sample catches of tuna several hours after they have been taken. Erythrocytes collected from living fish and kept in whole blood at ordinary refrigeration temperatures lasted as long as a week.

This stability, coupled with the relatively large amounts of blood obtained from single fish, proved of great basic value in the studies to be reported. Tuna bloods did not seem to clot to the extent that many other fish bloods do, relatively small clots being formed in a given sample. Two-percent washed cell suspensions were prepared fresh each day, (even though such preparations often kept well for 3 or 4 days in the refrigerator). They were made by washing the erythrocytes contained in a half milliliter of whole blood 3 or more times in at least 15 ml. of 1.5 percent sodium-chloride solution. This salt solution proved so satisfactory that no comparative studies were made on other types of solutions.

Agglutination tests were made by putting 2 drops of 2-percent cell suspension together with 2 drops of suitably diluted antiserum in test tubes (10 mm. X 70 mm.), allowing them to stand at room temperature for 15 minutes, centrifuging at 1,000 r.p.m. for 30 seconds, and observing the degree of agglutination upon resuspending. The conventional method of recording by estimating degree of agglutination in terms of pluses was employed, with 4+

representing essentially complete agglutination and 3+, 2+, and 1+ lessening degrees to 0 or no agglutination.

Two general kinds of antibodies were employed. Those found in "normal" bovine and sheep serums, and those found in the serums of individual rabbits previously injected with the cells of one or another kind of fish. The reactions of these serums with fresh tuna cells were surveyed (Cf. table 7) preliminary to further tests. This survey showed marked variations among the specificities and titers of the serums, and indicated that antibodies capable of reacting with fresh tuna cells are not universally present in rabbit serums at dilutions of 1 in 32 or 1 in 50. Frozen whole bloods, diluted 1 in 4, were used in the preparation of the serums, and a variety of injection schedules, all in use at one laboratory or another, was followed. No general conclusions can be reached as to which of these was the most successful. As fresh cells of the species used for immunization were usually not available at the time of collection, cells of other species, capable of cross-reacting with the serums, were often used in preliminary titration. All serums were kept frozen without the addition of preservatives when not in use, some being thawed and refrozen as much as a dozen times without apparent change in titer and specificity. Serums were readily transported to and from the Hawaiian Islands in an insulated cloth picnic bag containing dry ice. Characteristics of the serums that were used the most extensively in these studies are given below; additional properties are described where they specifically pertain to the experiments described in this paper.

Anti-yellowfin tuna 2 - immunization with aliquots of whole blood of a single yellowfin tuna (Neothunnus macropterus Temminck and Schlegel) collected by P.O.F.I. at Canton Island, June, 1951.

Anti-oceanic skipjack - immunization with aliquots of whole blood of a single oceanic skipjack (Katsuwonus pelamis Linnaeus) collected by P.O.F.I. at Christmas Island, June, 1951.

Anti-albacore 10 - immunization with aliquots of the whole bloods of four albacore (Germo alalunga Gemlin) collected by E. K. Holmberg off the Oregon coast, July, 1952.

Anti-albacore 14 - immunization with aliquots of the whole blood of four albacore collected as above.

Anti-mackerel - immunization with aliquots of the whole bloods of two Pacific mackerel (Pneumatophorus japonicus diego Ayres) collected at Santa Barbara, June, 1951.

Normal bovine serum - obtained through the courtesy of Lucian Sprague and Dr. Clyde Stormont. Sample C78-2582-2779-1/20/53.

Normal sheep serum - purchased from the Cappel Laboratories, West Chester, Penn.

Human serum - commercial anti-A and anti-B typing serums obtained from the Hyland Laboratories, Los Angeles, Calif.

The fresh Hawaiian fish referred to in this paper were obtained from several sources during the author's stay at the Hawaii Marine Laboratory. The single little tunny or kawakawa (Euthynnus yaito Kishinouye) was taken by Lester Zukeran while surface trolling near the entrance of Kaneohe Bay. The single yellowfin or ahi (Neothunnus macropterus Temminck and Schlegel) was taken by Georges Gilbert while surface trolling near the entrance of Kaneohe Bay, Oahu. The single wahoo or ono (Acanthocybium solandri Cuvier and Valenciennes) was taken by Georges Gilbert about one mile off Diamond Head, Oahu. The oceanic skipjack or aku (Katsuwonus pelamis Linnaeus) were taken commercially by Harry

Yagi, Georges Gilbert, and the crew of the Venus during 1 day's fishing approximately 30 miles off Diamond Head in the vicinity of the Penguin Banks.

Absorptions of serums were performed by mixing 1 to 2 ml. of a suitably diluted serum with 0.1 ml. of packed erythrocytes in a small centrifuge tube. The cells in these mixtures were kept suspended by hand or machine agitation for periods extending to 20 minutes at room temperature. After this time the absorbing cells were removed by centrifugation. One absorption generally sufficed to remove the antibodies for the antigens on the cells involved.

EXPERIMENTS WITH INTACT ERYTHROCYTES

Individual variation in skipjack antigens. Ten individual skipjack were found to fall into four categories which were distinguishable by variations in the specific affinities of their erythrocytes for antibodies in various serums. Fish of the first category (fish Nos. 11, 13) carried an antigen (1) with a relatively strong affinity for antibodies in various rabbit antiserums prepared against the blood of yellowfin tuna, albacore, skipjack, and white croakers. Fish of the second category (fish Nos. 12, 14, 17, 18, 19) had an antigen (2) with a relatively strong affinity for "natural" antibodies occurring in certain "normal" serums (human, bovine and sheep). Fish of the third category (fish Nos. 10, 15) had both antigens 1 and 2 on their cells. One fish (No. 16) of the fourth category had neither of the antigens (1 and 2) noted above. For convenience, the occurrence of antigens 1 and 2 is shown in all the tables that follow by superscripts following the numbers of individual fish.

Table 1 shows that fish carrying antigen 1 (Nos. 10, 11, 13, 15) differ markedly from those not carrying this antigen with respect to the degree to which their cells were agglutinated by selected rabbit antiserums. The numbers in the line labeled totals were simply obtained by adding the numerical estimates of the individual reactions above and are presented as a convenient single number for comparative purposes. (The reactions of fish No. 16 appear to involve an antigen other than those described in the text as 1 and 2).

Table 2 shows the results of absorbing 1 in 50 dilutions of albacore No. 10 and oceanic skipjack antiserums with cells of selected fish (the technique of absorption is described in the section on materials and methods). These results are in harmony with the concept that fish Nos. 10, 11, 13, and 15 carry an antigen 1 that distinguishes them from the other fish studied. The white croaker and mackerel antiserums shown in table 7 were also absorbed at 1 in 50 dilution using cells of Nos. 12 and 13. In both cases the No. 12 cells left antibodies for No. 13 cells, while No. 13 removed antibodies for both fish. Antigen 1 appears from these data to have a greater ability to combine with the heterogeneous antibody populations used in its detection than does antigen 2. This suggests a structural relationship between the two antigens that is similar to those classically described for the A_1 and A_2 subgroups of human A antigen.

In addition to the several reactions noted above, a 1 in 4 dilution of the normal serum of fish No. 12 (with antigen 2), weakly but definitely agglutinated the cells of fish Nos. 10, 11, 13, and 15. This reaction, shown in table 3, was confirmed by a duplicate test and by the use of undilute 12 serum. The serum of fish 17 (also with antigen 2) gave, in the duplicate test, an indication of agglutination with the cells of fish Nos. 10 and 11, but the reaction was too slight to be systematically studied. Although alternative interpretations are also possible, this observation is in agreement with the concept that antigen 1 occurs on the cells of these fish.

Populations of antibodies with relatively strong affinities for an antigen, 2, on the cells of fish Nos. 10, 12, 14, 15, 17, 18, and 19 were discovered in the normal bovine, sheep, and human blood grouping serums described in the section on materials and methods. Table 4 shows the reactions of selected skipjack cells with these serums.

Table 1.--Reactions of cells of individual skipjack with selected rabbit antiserums

Serum Dilutions:	Cells									
	$10^{1,2}$	11^{1}	12^{2}	13^{1}	14^{2}	$15^{1,2}$	16^{0}	17^{2}	18^{2}	19^{2}
1:50										
Albacore No. 10	3	3	±	3	±	3	1	±	1	±
Oceanic skipjack	4	4	2	4	1	3	1	±	1	1
Pacific mackerel	3	4	1	4	1	3	1	±	3	1
1:100										
Albacore No. 10	2	1	±	2	0	2	±	0	±	0
Oceanic skipjack	2	3	1	3	1	2	±	0	±	±
Pacific mackerel	3	2	±	2	1	3	±	±	1	±
1:200										
Albacore No. 10	±	±	0	1	0	±	0	0	0	0
Oceanic skipjack	1	±	0	1	0	1	0	0	0	0
Pacific mackerel	1	±	0	1	0	1	0	0	0	0
TOTALS	19.5	18.5	5.5	21.0	4.5	18.5	4.5	4.0	7.0	3.5

Table 2.--Results of absorbing rabbit antiserums with cells of different individual skipjack

Absorbed serums	Cells									
Albacore No. 10 absorbed with:	$10^{1,2}$	11^{1}	12^{2}	13^{1}	14^{2}	$15^{1,2}$	16^{0}	17^{2}	18^{2}	19^{2}
Cells of 12^{2}	3	3	0	2	0	2	0	0	0	0
Cells of 13^{1}	0	0	0	0	0	0	0	0	0	0
Oceanic skipjack absorbed with:										
Cells of 11^{1}	0	0	0	0	0	0	0	0	0	0
Cells of 14^{2}	3	3	0	3	0	3	0	0	0	0

Table 3.--Isoagglutination reactions among individual skipjack

Cells (Superscripts show antigens)	Serums, diluted 1 in 4									
	10	11	12	13	14	15	16	17	18	19
$10^{1,2}$	0	0	±	0	0	0	0	0	0	0
11^{1}	0	0	±	0	0	0	0	0	0	0
12^{2}	0	0	0	0	0	0	0	0	0	0
13^{1}	0	0	+	0	0	0	0	0	0	0
14^{2}	0	0	0	0	0	0	0	0	0	0
$15^{1,2}$	0	0	+	0	0	0	0	0	0	0
16^{0}	0	0	0	0	0	0	0	0	0	0
17^{2}	0	0	0	0	0	0	0	0	0	0
18^{2}	0	0	0	0	0	0	0	0	0	0
19^{2}	0	0	0	0	0	0	0	0	0	0

Table 4.--Reactions of individual skipjack cells with normal serum

Cells		Serum Dilutions					
		1:2	1:4	1:8	1:16	1:32	1:64
		Human anti-A (Hyland Laboratories)					
Human A		4	4	4	3	3	2
Skipjack	11^{1}	±	0	0	0	0	0
	12^{2}	4	2	1	±	0	0
	13^{1}	1	±	0	0	0	0
	14^{2}	4	4	2	1	0	0
		Human anti-B (Hyland Laboratories)					
Human B		4	4	3	2	1	±
Skipjack	11^{1}	±	0	0	0	0	0
	12^{2}	4	2	1	±	0	0
	13^{1}	1	±	0	0	0	0
	14^{2}	4	2	1	±	0	0
		Normal bovine					
Skipjack	$10^{1,2}$	3	2	2	1	1	±
	12^{2}	4	4	3	2	1	1
	13^{1}	2	1	±	0	0	0
	18^{2}	4	3	2	1	1	±
		Normal sheep					
Skipjack	$10^{1,2}$	4	3	2	1	±	0
	12^{2}	4	4	4	2	1	+
	13^{1}	3	1	+	0	0	0
	18^{2}	4	3	2	2	1	±

Table 5 shows the results of absorption tests of normal bovine serum with the cells of selected skipjack. This table shows the reactions of normal bovine, 1 in 8 dilution, and of normal bovine, 1 in 4 dilution, absorbed with selected cells. The letters a, b, and c after the absorptions with No. 13's cells show the results of separate absorptions. The observations conform with the concepts that fish Nos. 10, 12, 14, 15, 17, 18, and 19 carry an antigen (2) on their cells that reacts strongly with antibodies in normal bovine serum, and that fish 11, 13, and 16 lack this antigen.

Table 6 shows the results of absorbing a mixture of normal sheep serum and albacore No. 10 antiserum in final concentrations of 1 in 4 and 1 in 50 respectively. (Note that sheep serum was used because the available bovine serum supply was exhausted during the study. Table 4 shows that the sheep serum contains antibodies with affinities similar to those in bovine serum, a point borne out by the absorptions in table 6). The observations recorded conform with the concept that fish Nos. 10 and 15 have both antigen 1 and 2 on their cells, fish Nos. 11 and 13 have only antigen 1 on their cells, fish Nos. 12, 14, 17, 18, and 19 have only antigen 2 on their cells and fish No. 16 has neither antigen 1 nor 2 on its cells. This concept must of course be taken only as a guide to further studies, involving larger series of fish.

Comparative study of different species. As noted above, single individuals of the following species of fish were available for serological study; yellowfin tuna or ahi, little tunny or kawakawa, and wahoo or ono. These individuals were not all available at the same time, nor concurrently with the skipjack, so that complete use of materials for comparative study could not be made. Table 7 shows the reactions of the cells of various species with a series of antiserums, prepared as described in materials and methods. Consideration of this table shows several marked contrasts in the reactions of cells of different fish with the same antiserums. Of particular interest are the albacore antiserums, for one alternative explanation of their reactions is the possibility that individual variations occur in albacore antigens. Those tests marked with an asterisk (*) were run at 1 in 50 serum dilution rather than at 1 in 32. The cells of the wahoo had become quite fragile at the time of the test and the readings may not prove to be reliable.

Titrations were run on certain antiserums using the cells of a yellowfin tuna and a little tunny. These antiserums are marked with a "dagger" (†) in table 7. Differential reactions between the two species, paralleling those recorded in table 7, could still be observed at dilutions of 1 in 200 and 1 in 400 for the serums concerned.

As the little tunny is similar in its reactions to skipjack Nos. 10, 11, 13, and 15, it seems likely that these two species vary intraspecifically in similar ways, a point that would not be unexpected considering their rather close evolutionary relationship. Absorption of bovine and human typing serums confirmed the antigenic distinctiveness of the yellowfin and little tunny.

EXPERIMENTS WITH FROZEN HEMOLIZED WHOLE TUNA BLOODS

Natural antibodies. Research on tuna bloods was begun at a time when fresh tuna erythrocytes were not available, and when the author had received a variety of samples of frozen whole tuna bloods from the sources credited at the beginning of this paper. Considerable effort was therefore made to develop techniques that would permit the detection of individual differences in frozen, hemolized whole blood. As reported in earlier papers (Cushing 1952, a and b), individual variations in the natural antibody content of these bloods were discovered, notably with respect to the agglutination of human type-B cells. The validity of these observations was confirmed by the discovery of individual variations in the natural antibody content of fresh, unfrozen oceanic skipjack serums obtained in Hawaii. These are shown in table 8.

No agglutinins for human cells or sheep cells were found in a sample of 24 frozen albacore bloods, collected July 24 to 26, 1952, off

Table 5.--Results of absorbing normal bovine serum with cells of different individual skipjack

Antiserums	Cells									
	$10^{1,2}$	11^{1}	12^{2}	13^{1}	14^{2}	$15^{1,2}$	16^{0}	17^{2}	18^{2}	19^{2}
Bovine 1 in 8 not absorbed	4	0	3	±	3	3	0	2	3	1
Bovine 1 in 4 12 abs.	±	0	0	±	0	1	0	0	0	0
13 abs.-a	4	0	4	0	4	3	0	4	4	2
13 abs.-b	4	0	4	0	4	4	0	4	3	2
13 abs.-c	3	0	4	0	4	3	0	4	4	2
14 abs.	0	±	0	0	0	±	0	0	0	0

Table 6.--Absorption of serum mixture containing normal sheep serum, 1 in 4, and albacore 10, 1 in 50

PART A

The results of these absorptions are discussed in the text. Reciprocal tests among 10, 11, 12, and 16 were run in duplicate. Cells of fish 19 were becoming too fragile to work with readily, as shown by their tendency to hemolize (H).

Antiserums	Cells									
	$10^{1,2}$	11^{1}	12^{2}	13^{1}	14^{2}	$15^{1,2}$	16^{0}	17^{2}	18^{2}	19^{2}
Nonabsorbed	3	3	4	3	4	3	0	4	4	H
Absorbed by 10	0	0	0	0	0	0	0	0	0	0
" " 11	3	0	4	0	4	3	0	4	3	1
" 12	3	2	0	3	0	3	0	0	0	0
" " 16	3	2	2	4	3	3	0	2	2	H

PART B

The absorptions in this part, while supporting the concepts of antigenic relationships described in the text, were made at a time when the red cells used were becoming increasingly fragile. (Note H reactions). This fact probably accounts for the relative weakness of some of the reactions observed, and for the inconsistent reaction of serum absorbed by fish 14 with the cells of fish 11.

Antiserums	Cells									
	$10^{1,2}$	11^{1}	12^{2}	13^{1}	14^{2}	$15^{1,2}$	16^{0}	17^{2}	18^{2}	19^{2}
Absorbed by 13	2	0	3	0	4	3	0	2	2	H
" " 14	1	0?	0	2	0	1	0	0	0	0
" " 15	0	H	0	0	0	0	0	0	0	H
" " 16	4	H	3	1	4	1	H	1	2	H

Table 7.--Reactions of cells of various species of fish with selected antiserums

Antiserums (1 in 32)	Cells				
	Yellowfin	Little tunny	Wahoo	Oceanic skipjack 12[1]	Oceanic skipjack 13[2]
Yellowfin 1	4	±	1	±*	0*
Yellowfin 2	4	0	1	0*	±*
Yellowfin 3:4B	4	0	±	0*	0*
Yellowfin 8	4	0	±	0*	0*
Yellowfin 9	2	±	±	0*	0*
Albacore 10	1	4	2	±*	3*
Albacore 14	4	0	1	0*	0*
Skipjack	±	4	1	2*	4*
Mackerel	4	4	3	1*	4*
White croaker	4	4	±	4	4
Shiner seaperch	4	0	0	±	0
Sheep hemolysin (1 in 8)	4	0	±	0	1
Normal bovine	4	4	---	3	±
Normal sheep	4	±	---	1	0
Human anti-A	3	0	±	±	0
Human anti-B	2	0	0	±	0

Table 8.--Agglutination of human erythrocytes by fresh skipjack serums

Cells	Serums, diluted 1:4								
	$10^{1,2}$	11^{1}	12^{2}	13^{1}	14^{2}	$15^{1,2}$	16^{0}	17^{2}	18^{2}
Type A	0	0	±	0	±	0	±	0	0
Type B	3	±	3	4	2	4	3	1	2
Type O	0	0	±	0	±	0	±	0	0

the Oregon coast by E. K. Holmberg on the tuna troller Scarab. This observation suggests at least the possibility of interspecific differences with respect to the occurrence of natural agglutinins in bloods of some species of tuna. (Alternative explanations such as seasonal variations in antibody titer and parasitic infestations are of course possible. These problems are currently being studied in this laboratory with the assistance of George Durall, using as a model an isoantibody system discovered this year in a local population of catfish (manuscript in preparation).

Human "type-A" substance in frozen tuna blood. Studies conducted at Santa Barbara with the assistance of Barbara Drake and Lucian Sprague have supplied evidence of a substance in tuna blood resembling the human A blood type antigen. Table 9 shows the results of a series of slide agglutination tests that give evidence of a specific inhibition of human anti-A typing serum by the centrifuged (5,000 r.p.m. for 20 minutes) whole bloods of yellowfin tunas. All tests were performed by mixing 1 drop of frozen tuna blood, previously diluted 1 in 4 with 1 percent saline solution, with 1 drop of serum dilution. Cells were added to this mixture after it had stood for 15 minutes at room temperature. Readings were made 15 minutes after this step. It will be noted that some of the tuna bloods contain natural agglutinins for human B cells but that only one (fish 204) is capable of agglutinating human A cells as well. One fish, 209, appears to lack A inhibitor, but whether this is indicative of individual lack of A antigen is not known.

The experiments recorded in table 10 show that "blocking" ("incomplete," "inhibiting") antibodies in tuna blood are not the cause of the inhibition noted, for the absorption of tuna blood with human A cells did not reduce the inhibiting factor. Here the blood of a single tuna (207) was diluted 1 in 4 with 1 percent saline and absorbed with human cells. A drop of absorbed blood was placed on a slide with a drop of serum dilution and allowed to stand for 15 minutes after which time a drop of cell suspension was added. Agglutinations were read after 15 minutes. The erythrocytes were washed in 1 percent saline solution after being used for absorption and were then tested with typing serums to see if any A antigen had been absorbed by them. Negative results were obtained in these tests.

The inhibition was found to be partially associated with material that failed to pass through a Sweeny bacterial syringe filter. This material was presumably fragments of stroma. This presumption is supported by observations that washed stroma causes specific inhibition and also specifically absorbs anti-A from a mixture of anti-A and anti-B serums. (These last two observations are of a preliminary nature in that the relatively low titers of inhibitor and available antiserums made it difficult to obtain markedly contrasting preparations).

Table 9.--Specific inhibition of human anti-A typing serum by tuna blood

Fish numbers:	Human type-A cells plus dilutions of human anti-A serum				Human type-B cells plus dilutions of human anti-B serum			
	1/2	1/4	1/16	Saline	1/2	1/4	1/16	Saline
Saline	4	3	0	0	4	3	1	0
202	2	0	0	0	4	3	0	0
203	2	0	0	0	4	4	0	0
206	2	0	0	0	4	4	1	0
207	3	0	0	0	4	4	1	0
214	2	0	0	0	4	4	1	0
200	3	0	0	0	4	4	1	2
201	2	0	0	0	4	4	0	1
208	3	0	0	0	4	4	1	1
209	4	3	0	0	4	4	4	3
210	3	1	0	0	4	4	2	2
211	2	0	0	0	4	4	1	2
212	2	0	0	0	4	4	3	2
213	3	0	0	0	4	4	1	2
216	3	1	0	0	4	4	1	1
217	2	0	0	0	4	4	1	1
204	4	4	4	2	4	4	4	3

Table 10.--Effect upon inhibition of absorption of blood of a yellowfin tuna with human erythrocytes

	Reactions of human A cells with dilutions of anti-A serum combined with tuna blood absorbed as shown.				Reactions of human B cells with dilutions of anti-B serum combined with tuna blood absorbed as shown.			
	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16
Saline	3	3	2	0	3	3	3	1
Non-absorbed tuna blood	1	0	0	0	3	3	3	1
A absorbed tuna blood	1	0	0	0	3	3	3	1
B absorbed tuna blood	1	0	0	0	3	3	3	1
0 absorbed tuna blood	1	0	0	0	3	3	3	1

Observations were also made upon the reactions of fresh tuna erythrocytes with human anti-A and anti-B typing serums. Table 7 shows that the little tunny and oceanic skipjack show very little reactivity with these serums, while the reactions of the yellowfin are much more marked. However, as both anti-A and anti-B serums react almost equally well, normal antibodies must be involved that are not specifically related to the human blood types. Absorption of anti-A serum with yellowfin cells, while removing all agglutinins for these cells, was not observed to reduce the titer of the anti-A serum with respect to human A cells. The preliminary observations on stroma noted above and the recollection of the relationships among the human A subtypes shows that this is not a conclusive observation. Further observations upon fresh yellowfin cells could not be made, so that the nature of the A-like substances on tuna cells still remains to be elucidated (Cf. Cushing and Sprague, 1953, for an earlier discussion of this problem). A final observation in this connection is that the antiserums in table 7 that were titrated (/) were not able to agglutinate human A cells in spite of their reactivity for the cells of tuna.

Tuna stroma. Much attention was given to the preparation and agglutination of washed tuna stroma in the hope that these might be useful in studies where fresh red cells could not be obtained. However, it was found difficult to obtain consistent preparations and to achieve agglutinations to any usable degree. As a result of many observations the conclusion was reached that future efforts should be made to work with intact erythrocytes rather than to develop techniques using frozen whole bloods in which the cells had been hemolized.

Preservation of intact erythrocytes. The above conclusion diverted research from frozen tuna blood to efforts to preserve intact erythrocytes by freezing in glycerol. This method has been successfully applied to the preservation of human erythrocytes by Chaplin and Mollison (1953) in England. While fresh tuna bloods were not available, it was possible (with Mrs. Elyse Beaver) to show that small aliquots (appx. 1 to 3 ml.) of the cells of shiner seaperch (Cymatogaster aggregata Gibbons) could be preserved by a modification of this technique, and that the cells of other species also gave promising results. At present it may be concluded that the glycerol-freezing technique should be investigated whenever it becomes desirable to make and preserve large scale collections of tuna blood from diverse areas.

Forssman antigen. Three anti-tuna serums were tested for their ability to hemolize sheep cells in the presence of guinea pig complement. The hemolytic titers observed after 15 minutes at 37°C. were as follows: anti-yellowfin 1, 1:1280; anti-oceanic skipjack, 1:640; anti-pacific mackerel, 1:5120.

Tuna antigens occurring in white croakers and shiner seaperch. Cells of the shiner seaperch (Cymatogaster aggregata Gibbons) were found to be agglutinated by anti-yellowfin tuna serum 2 to a titer of 1:4096. Conversely, cells of the white croaker (Genyonemus lineatus Ayres) were found to be agglutinated to a titer of 1:400 by the anti-oceanic skipjack serum already referred to in this paper. Shiner seaperch cells were also weakly agglutinated by this second serum, but this agglutinin could be absorbed, leaving the white croaker agglutinin intact. Table 11 shows the results of absorbing a mixture of anti-yellowfin 2 and anti-skipjack serum with the washed stroma of individual yellowfin and skipjack. The preliminary absorption of skipjack antiserum with shiner seaperch cells was necessary to remove the low-titer agglutinins for these cells. The results show that yellowfin tuna and skipjack are actually distinguishable by two antigens found on white croaker and shiner seaperch cells respectively.

As table 7 shows, variations exist among the species of tuna examined with respect to reactions with anti-albacore and anti-yellowfin tuna serums; therefore, the two antigens under discussion would seem to be of potential interest in evolutionary investigations along the lines discussed in Cushing and Sprague, 1953. The possibility also exists that antigens of such wide distribution among diverse species may be of value in searching for individual antigen variations within single species of fish. For example,

work with Donald Shaw on the absorption of anti-skipjack serum with the cells of individual white croakers revealed minor differences in the absorptive power of the cells of individual fish. These differences were accentuated when cells of the walleye surfperch (Hyperprosopon argenteum Gibbons) were included as test antigen. The relationships among the antigens involved are complex, from which it is apparent that interspecific combinations of different species of fish with respect to immunization, absorption, and testing may be profitably manipulated in the study of erythrocyte antigens. Table 12 shows that the absorption of anti-skipjack serum varies with the cells of individual fish. This variation is revealed by comparing the residual agglutination titers for the cells of a single walleye surfperch. Two "types" of white croaker are indicated on the basis of the amount of agglutinin remaining.

The compexity of this relationship is further shown by the fact that the two anti-albacore serums (Nos. 10 and 14) failed to agglutinate either white croaker or shiner seaperch cells, while agglutinating the cells of skipjack and other tuna to varying degrees (review table 7).

A final point is that the agglutination of white croaker cells by anti-skipjack serum can be inhibited through the use of previously frozen whole skipjack blood, thawed and centrifuged free of cellular debris. This observation shows that it may be possible to utilize inhibition techniques in investigations where only frozen whole bloods can be obtained.

DISCUSSION

The observations and experiments reported above show that it is very probable that serological techniques can be applied profitably to the study of racial and specific variation in tunas. Not only do individual variations exist among the erythrocyte antigens of single species (oceanic skipjack) but species variations also exist among the yellowfin, albacore, little tunny, and skipjack. In addition, the bloods of tunas seem to be easy to collect and to work with. Further, it is apparent that not only can specific immune serums be prepared against tuna blood, but that systematic research may develop the usefulness of "normal" antibodies as reagents for distinguishing individual variations as has been done, for example, with eel serum in human blood-group studies (Race and Sanger, 1954) and with bovine serum in chicken blood-group studies (Briles, Briles, and Irwin, 1951).

The widespread occurrence of heterogenetic antigens among fish also offers various opportunities for further investigation of serological variations within tunas, as is shown through examples of the use of inhibition and absorption techniques. A review of the various data presented in this paper suggests considerable complexity among the specific relations of the heterogenetic antigens so far detected, and that any detailed study of a single group should reveal information of general interest concerning the evolution of these antigens among fish in general.

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Table 11.--Absorption of a mixture of equal volumes of anti-yellowfin 2 and shiner seaperch absorbed anti-skipjack (final dilution 1 in 16)

Not absorbed: (mixture)		Absorbed with yellowfin stroma:		Absorbed with skipjack stroma:	
Seaperch	White croaker	Seaperch	White croaker	Seaperch	White croaker
4	4	0 (fish 706)	3	4 (fish 100)	±
(anti-yellowfin 2, 1:16)					
4	0	0 (fish 824)	4	4 (fish 105)	0
(anti-skipjack, 1:16)					
2	4	0 (fish 710)	4	4 (fish 107)	0
(seaperch absorbed anti-skipjack)					
0	4				

Table 12.--Individual heterogeneity of white croaker antigens

		Dilutions of anti-skipjack serum				
Treatment of serum:	Cells	1/10	1/20	1/40	1/80	1/160
unabsorbed	walleye surfperch	4	4	4	4	3
	white croaker R-27	4	4	4	4	3
	white croaker R-23	4	4	4	4	3
absorbed with	walleye surfperch	0	0	0	0	0
walleye surfperch	white croaker R-27	4	4	4	4	3
	white croaker R-23	4	4	4	4	3
absorbed with R-27	walleye surfperch	4	4	2	1	0
	white croaker R-27	1	0	0	0	0
absorbed with R-26	walleye surfperch	3	1	±	0	0
	white croaker R-27	1	±	0	0	0
absorbed with R-25	walleye surfperch	4	4	3	2	±
	white croaker R-27	±	0	0	0	0
absorbed with R-24	walleye surfperch	3	1	0	0	0
	white croaker R-27	1	0	0	0	0
absorbed with R-23	walleye surfperch	4	2	1	0	0
	white croaker R-23	±	0	0	0	0
absorbed with R-21	walleye surfperch	4	3	2	1	±
	white croaker R-23	±	±	0	0	0
absorbed with R-18	walleye surfperch	4	3	2	1	±
	white croaker R-23	±	0	0	0	0

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